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Free radical-mediated reperfusion injury: A selective review

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Summary Tissue damage as a consequence of ischemia is a major medical problem in an industrialized society. Whereas the conventional view has attributed this injury process to ischemia itself, recent studies have found that a variable, but often substantial proportion of the injury is caused by toxic oxygen metabolities that are generated from xanthine oxidase at the time of reperfusion. This mechanism was first identified and characterized in a model of moderately mild partial vascular occlusion in the feline small intestine. Strikingly similar mechanisms have been subsequently confirmed as the basis for ischemia/reperfusion injury in the stomach, pancreas, liver, skin, skeletal muscle, heart, lung, kidney and central nervous system. The potential for clinical application of this concept is related primarily to that proportion of the total post-ischemic injury that is due to this reperfusion mechanism, set against the proportion due to ischemia itself. Ironically, in clinical cases of intestinal ischemia the reperfusion component appears to be proportionately small, and the potential for treatment of ischemic bowel disease is correspondingly limited. On the other hand, there is reason to expect that the ablation of free radical-mediated reperfusion injury, something that can be readily achieved through non-toxic means, may provide substantial benefit for the treatment of ischemic renal disease, myocardial infarction, stroke, cardiac arrest, and of organs preserved for transplantation.

Ischemic vascular disease is the underlying cause of stroke, heart attack, renal failure, a number of other common diseases. It even constitutes a major component of the long-term damage sustained by normal tissues as a consequence of radiation injury. Recent advances in our understanding of the fundamental mechanisms of post-ischemic injury, however, suggest that a major proportion of the injury that appears as a consequence of ischemia is sustained at the time of reperfusion. This means that, in many clinical situations, the damage that is often considered to be irreversible may not yet have been sustained. Furthermore, the mechanism that mediates this injury is a remarkably ubiquitous one that lends itself readily to therapeutic intervention.

The fundamental mechanism

The discovery of the endogenous free radical scavenger superoxide dismutase (SOD) by McCord and Fridovich in 1969, led to a period of rapid progress in the understanding of free radical-mediated tissue injury. Much of this work has focused on superoxide production in neutrophils by a membrane-bound NADPH oxidase (McCord, 1983; Roy, 1982). However, McCord and Fridovich (1968) had also found that superoxide could also be generated in other cells as a byproduct of the oxidation of hypoxanthine and xanthine by xanthine oxidase. Under nonischemic conditions the activity of xanthine oxidase in many tissues is low, but it increases rapidly after the onset of ischemia. This is due to the proteolytic removal of a peptide fragment from another enzyme, xanthine dehydrogenase, which is abundant in many tissues (Roy & McCord, 1983). The activation of xanthine oxidase by ischemia provides a potential mechanism for free radical generation in parenchymal tissues following ischemia.

Knowing that the intestinal mucosa contained particularly high levels of xanthine dehydrogenase (Batelli *et al.*, 1972), which was rapidly converted to xanthine oxidase under ischemic conditions (Roy & McCord, 1982; McCord & Roy, 1982), Granger *et al.* (1981) applied this principle to a previously described, standard model of ischemic mucosal injury in the cat small intestine (Ahren & Haglund, 1973). Using the osmotic reflection coefficient, a rather precise measure of capillary permeability, as an index of injury, they found that the administration of SOD prevented most of the injury caused by a one hour period of hypotensive perfusion (partial ischemia). It is particularly noteworthy that SOD was effective when administered near the end of the ischemic period, shortly prior to reperfusion. The injury previously thought to be ischemic, must therefore be taking place at the time of reperfusion. Because SOD is an extremely specific inhibitor of superoxide, with no other known biological function, this constituted the first demonstration of free radical-mediated reperfusion injury.

Largely due to subsequent developments, their paper constitutes the cornerstone of the field. Here, Granger *et al.*, proposed the mechanism that has provided the framework for most of the subsequent progress in this field (Figure 1). With the onset of ischemia, there is rapid proteolytic conversion of xanthine dehydrogenase to xanthine oxidase, and an accumulation of the substrate hypoxanthine due to the breakdown of adenine nucleotides (ATP). The oxidation of hypoxanthine to xanthine cannot take place, however, in the absence of oxygen. When this missing substrate is suddenly available in excess, as it is at reperfusion, this oxidation proceeds rapidly, with the parallel reduction of the molecular oxygen. This reduction results in the generation of superoxide radicals as a byproduct. Although somewhat cytotoxic themselves, these superoxides can then go on to form hydrogen peroxide, and secondarily the extremely cytotoxic hydroxyl radical through the Haber–Weiss reaction (Haber & Weiss, 1934). These toxic oxygen metabolites cause cellular damage primarily through the peroxidation of lipids in the membranes of the cell and the mitochondrion (McCord, 1983). It is presumably this membrane injury to the endothelial cell that was manifest as the increase in capillary permeability measured by Granger *et al.* (1981).

Since this original report, a number of subsequent studies have supported this hypothesis. In the cat small intestine, SOD was also found to protect the mucosa from histologic evidence of frank epithelial necrosis after three hours of partial ischemia (Parks *et al.*, 1982*a*). Moreover, allopurinol, a specific inhibitor of xanthine oxidase, provided equivalent protection. Subsequently, this laboratory has demonstrated that a similar mucosal permeability lesion (manifest by the leakage of labeled albumin into the bowel lumen) can be produced by either ischemia/reperfusion or by the perfusion of the lumen with hypoxanthine and xanthine oxidase, even in the absence of ischemia (Grogaard *et al.*, 1982). The latter injury could be prevented with SOD. Furthermore, pretreatment with either allopurinol or the hydroxyl radical scavenger dimethylsulfoxide (DMSO) blocked the capillary permeability injury seen after one hour of ischemia (Parks & Granger, 1983). This injury could also be mimicked in this model by the intraarterial infusion of hypoxanthine and xanthine oxidase. This damage was also prevented with SOD or DMSO (Parks *et al.*, 1984). Finally, both the capillary

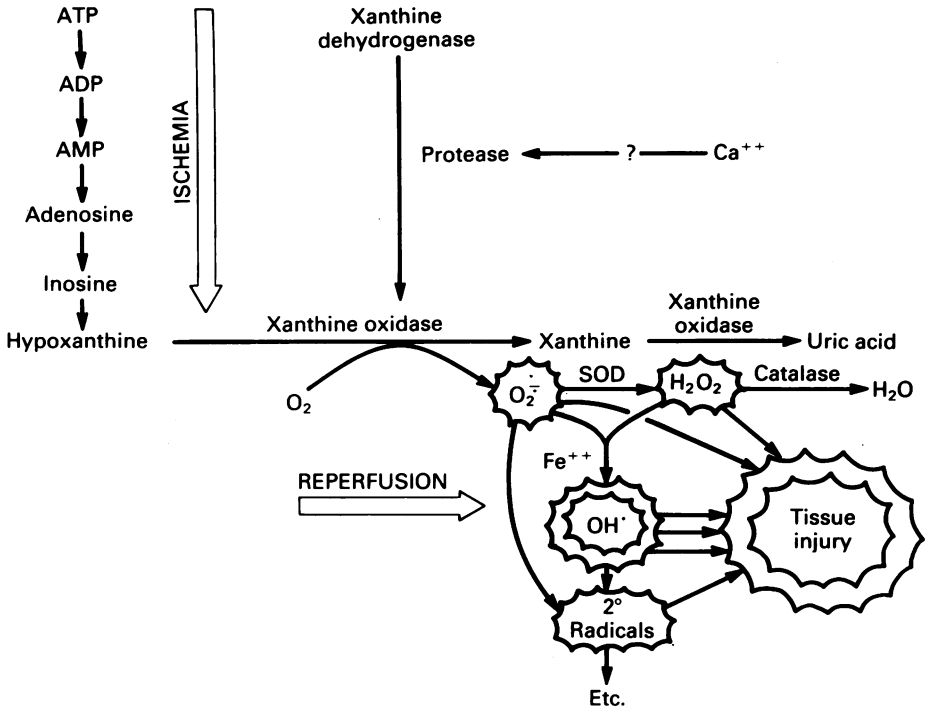

Figure 1 Mechanism of free radical generation in ischemic tissues at reperfusion. With the onset of ischemia, high energy phosphate compounds are broken down into lower energy adenine nucleotides, and finally into their purine bases, with the consequent accumulation of hypoxanthine. Under non-ischemic conditions, hypoxanthine is oxidized to xanthine, and xanthine is oxidized to uric acid by the enzyme xanthine oxidase. The low activity of this enzyme in most non-ischemic tissues suggests that this reaction is quite slow under normal conditions. With the onset of ischemia, however, there is rapid proteolytic conversion of xanthine dehydrogenase to xanthine oxidase. Therefore, both the substrate (hypoxanthine) and the activated enzyme (xanthine oxidase) are present in excess, but this oxidation reaction cannot proceed during the period of ischemia, due to the absence of molecular oxygen. This missing substrate is supplied suddenly, and to excess, at the moment of reperfusion, with the consequent rapid production of the superoxide free radical (O_2^-) as a byproduct. This species can cause cellular injury itself, or it can generate secondary radical species. The most important of these is the extremely reactive and highly toxic hydroxyl radical (OH·), which is generated by the iron-catalyzed reduction of H_2O_2 and O_2^-, a Fenton reaction described by Haber and Weiss (1934). (Modified from Granger *et al.*, 1981).

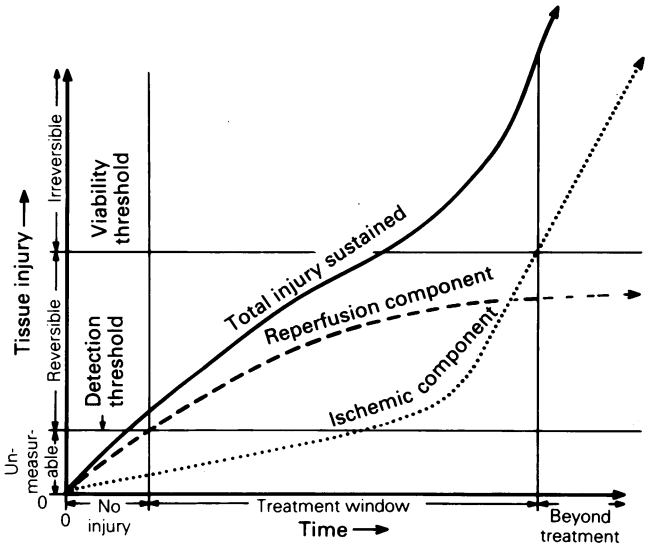

Figure 2 Ischemia/reperfusion injury. At the onset of ischemia, two separate process start, each with a different time course. One is the processes of tissue damage due to ischemia itself. The second is the biochemical changes described in the text which will lead, at the time of reperfusion, to reperfusion injury. Therapy directed at free radical-mediated reperfusion injury will only be effective when administered after a period of ischemia that corresponds to the therapeutic window, where the major component of the total injury is due to reperfusion. Although this window appears to vary from organ to organ, in many animal models of human disease, its apparent magnitude in many clinical situations renders this approach promising.

permeability seen after one hour, and the mucosal histologic injury seen following three hours of partial arterial occlusion was ablated by pretreatment with soybean trypsin inhibitor, a serine protease inhibitor that blocks the proteolytic activation of xanthine oxidase from xanthine dehydrogenase (Parks *et al.*, 1985). Although they constitute indirect evidence, obtained from the use of specific (SOD, catalase), somewhat specific (allopurinol, soybean trypsin inhibitor) and relatively nonspecific (mannitol, DMSO) inhibitors of particular biochemical processes, these data comprise an overwhelming body of circumstantial evidence for the above scheme as the primary mechanism of post-ischemic reperfusion injury (Parks *et al.*, 1983*a*).

There have been noteworthy negative results, however. Despite extensive efforts, we have been unable to demonstrate protection by either SOD or allopurinol in the cat or rat small intestine following varying periods of *total* vascular occulsion (Parks *et al.*, 1982*b*; 1983*a*). Indeed, more serious degrees of intestinal injury seem unimproved by manipulation of this free radical mechanism. We interpret these to indicate that this latter injury is more attributable to ischemia itself.

Application to the treatment of ischemic disease

In general, the utility of anti-free radical therapy for the treatment of tissue injury resulting from ischemia will probably be related primarily to the proportion of injury that is due to reperfusion, set against the proportion due to ischemia *per se*. Although this is still a very new field, preliminary indications of this utility are beginning to

emerge. Ironically, this approach is unlikely to benefit most patients suffering from ischemia of the *intestine*. These people usually die from an advanced transmural infarction, which is far more severe than the superficial mucosal lesion described above. This advanced injury appears to be due mostly to ischemia itself, and cannot be ameliorated substantially by treating the events at reperfusion. Only in young animals is there a suggestion that an injury leading to full thickness necrosis can be prevented with free radical scavengers (Oshima *et al.*, 1984). This has provided some basis for hope that the application of the above principles may prove to be fruitful in the treatment of neonatal necrotizing enterocolitis (Parks *et al.*, 1983; Bailey & Bulkley, 1987).

In the *stomach*, there is evidence that the superficial gastric mucosal ulceration seen in response to severe stress ('stress gastric ulceration') can be prevented by treatment with SOD (Itoh & Guth, 1985; Perry *et al.*, 1986). Studies in the isolated, perfused canine *pancreas* indicate that in models of acute pancreatitis, caused not only by ischemia, but also by gallstones or by the hyperlipidemia that mimicks that seen with alcohol ingestion, the injury is markedly ameliorated by pretreatment with either SOD or allopurinol (Sanfey *et al.*, 1984; Sanfey *et al.*, 1985). This had led us to speculate on the possibility that the final common pathway in acute pancreatitis could be the proteolytic activation of xanthine oxidase from xanthine dehydrogenase by a pancreatic protease, probably chymotrypsin (Sanfey *et al.*, 1985; Sanfey *et al.*, 1986). Studies in the rat liver have shown that much of the injury that has previously been attributed to liver ischemia may well be mediated by free radicals at reperfusion (Adkinson *et al.*, 1986). This is also supported by a study by Norstrom *et al.* (1985), which shows substantial protection of the rat liver from ischemic injury by pretreatment with allopurinol.

A series of studies in rat abdominal *skin* suggest an important role for this mechanism in post-ischemic skin injury. Both SOD and allopurinol substantially protect both island skin flaps and free flap transfers from necrosis following the ischemia necessitated by their creation (Manson *et al.*, 1983; Im *et al.*, 1984; Im *et al.*, 1985; Narayan *et al.*, 1985; Manson *et al.*, 1986). In these studies, biochemical assays of xanthine oxidase activity and the production of malondialdehyde as an indicator of lipid peroxidation also support the proposed mechanism.

Skeletal muscle ischemia is of particular interest to the peripheral vascular surgeon. Here, the increase in vascular permeability seen at reperfusion causes muscle swelling within a closed fascial compartment. This causes increased tissue hydrostatic pressure such that capillary perfusion ceases, secondary ischemia ensues, and the muscle undergoes ischemic necrosis. Korthuis *et al.* (1985) have demonstrated that this lesion is due to an increased capillary permeability that is blocked by anti-free radical treatment at reperfusion. Walker *et al.* (1987) have extended this to blocking the development of full-blown necrosis of the perfused canine gracilis muscle with free radical scavengers. Finally, Lee *et al.* (1987) have shown that SOD and catalase conjugated to polyethylene glycol (PEG-SOD) prevented the decrease in Ca^{++} uptake by the sarcoplasmic reticulum of rat skeletal muscle following three hours of tourniquet-induced hindlimb ischemia.

One of the earliest studies of the above mechanism was done by McCord in the rat *heart* (McCord *et al.*, personal communication). More recently, investigators interested primarily in the heart have moved rapidly to exploit the application of this concept, conceived and characterized in the intestine and other organs, to their own field. As a result, there is a rapidly increasing body of evidence to suggest that there may be clinical application of these principles to the treatment of global cardiac ischemia, as caused by the elective cardioplegic arrest that is necessary for cardic surgery during cardiopulmonary bypass (Jolly *et al.*, 1984; Burton *et al.*, 1984; Casale *et al.*, 1983; Stewart *et al.*, 1983; Shlafer *et al.*, 1982*a,b*), and of regional myocardial ischemia, specifically myocardial infarction (Chambers *et al.*, 1983; Gardner *et al.*, 1983). The quantitative impact of free radical injury modification was often large. One of the more interesting observations is that of Downey and his colleagues: allopurinol substantially reduced infarct size in the canine myocardium following embolization of the coronary artery, whether or not the embolus was subsequently removed to provide for gross reperfusion (Chambers *et al.*, 1983). These findings suggest that the events of ischemia and the events of reperfusion may not always be completely separated in time. Here, the two appeared to be taking place simultaneously. If this were true, therapy directed at free radical-mediated reperfusion injury could be beneficial as *primary* treatment of acute myocardial infarction. Moreover, this therapy might be expected to be effective after the onset of infarction. On the other hand, it is not yet clear whether or not the apparent reductions in infarct size are sustained over the long term.

One of the more exciting discoveries is that a substantial component of acute renal tubular necrosis, sustained as a result of renal ischemia, may be mediated by free radicals at reperfusion. Treatment by free radical ablation significantly ameliorates injury due to periods of warm ischemia (Hansson *et al.*, 1982; Hansson, 1983; Hansson *et al.*, 1987; Paller *et al.*, 1984; Baker & Corry, 1985; Parks *et al.*, 1983*b*).

Organ transplantation has advanced to the point that it is now standard treatment for chronic renal failure, with an over 80% success rate overall. Based on similar success rates, transplantation of the liver, the heart, and now even the heart and lung, have moved from being experimental curiosities to standard modes of therapy for end stage organ failure. Many feel that the routine use of pancreatic transplantation, already underway in diabetic kidney transplant recipients, will be available in the foreseeable future for the primary treatment of diabetes.

As the immunologic barriers have been better controlled, a major limitation in the use of these techniques has become the availability of organs for transplantation. As long as this need for organs exceeds supply, the effectiveness of organ preservation will remain a major factor limiting the success of transplantation. For example, many centres in the United States and Europe are forced to depend upon very wide catchment areas for kidney donors. This necessitates long delays between harvest and grafting. In many places, delays of 24–36 hours are common, and many otherwise good kidneys have to be discarded after longer periods of ischemic preservation. Unfortunately, current techniques of organ preservation are far from optimal. In fact, the reported incidence of acute renal failure is approximately 50% in many of the above hospitals. While the conventional approach to organ preservation has focused on minimizing the injury sustained during the period of ischemic cold storage by pump perfusion or by manipulation of the preservation medium, we have approached this problem from the standpoint of reperfusion injury. The beneficial effect in the kidney is substantial following periods of cold ischemia that mimick conditions of organ preservation (Koyama *et al.*, 1986). Animal studies in models of kidney, liver, heart, lung, and pancreas preservation already appear to confirm the application of this principle to the transplantation situation (for example, see Stuart *et al.*, 1985). Clinical trials are currently underway to evaluate the use of the SOD or allopurinol in cadaveric renal transplantation. It is not unreasonable to hope that the use of this approach will substantially increase the number of usable organs and significantly improve the overall results or organ transplantation in man.

Perhaps the most exciting potential for therapy directed at toxic oxygen metabolites at reperfusion is in patients who would develop brain damage or brain death due to *cerebral ischemia* from cardiac arrest or stroke. Conventionally, the

brain is thought to be limited to a 4–5 minute period of total ischemia before irreversible injury takes place. Cardiopulmonary resuscitation after such a period of ischemia is not worthwhile, even if cardiac function is restored, because of irreversible brain injury. If much of this brain damage does not take place until the time of resuscitation, the therapeutic and even medicolegal ramifications would be profound. Preliminary studies in gerbils suggest just such a possibility. Beckman and his colleagues (1986) have found that treatment with PEG-SOD substantially extends the period of total cerebral occlusion that can be tolerated without the development of fatal seizures shortly after reperfusion.

Unresolved problems

As this field has expanded, the approach that was successfully employed in the feline small intestine has been applied to a wide variety of organs, in animal models of important human diseases. Clinical trials are underway, or planned in the areas of renal transplantation, acute pancreatitis, elective cardioplegia for cardiac surgery, and perhaps somewhat prematurely, in acute myocardial infarction. At the same time, however, a number of fundamental scientific issues need to be resolved. Methods of free radical detection are still relatively primitive (Bulkley *et al.*, 1987) and often indirect measures are employed. The bulk of the information reviewed above constitutes circumstantial evidence that has been obtained using inhibitors of varying specificity. In the future, it will be important to combine radical detection techniques with the use of agents that perturb the process.

Another issue is the exact role of leukocytes in producing reperfusion injury. Undoubtedly, neutrophils play a role, both as generators of superoxide, and as agents of tissue injury (Jolly *et al.*, 1984). However, they cannot account for the xanthine oxidase system that initiates the process, as no one has yet been able to demonstrate xanthine oxidase activity in neutrophils (Jones *et al.*, 1985). My own belief is that the primary injury, illustrated in Figure 1, takes place within the endothelial cell. This would explain the uniformity of this mechanism in a variety of organs, as endothelial cells are ubiquitous. This initial endothelial lesion is then amplified by secondary infiltration of neutrophils, attracted to the site of injury. This degree of amplification would vary greatly from organ to organ, as does the susceptibility to neutrophil injury. This hypothesis, while entirely consistent with the available data, remains to be confirmed.

The major issue remains the clinical applicability to human disease, which appears to be related to the proportion of the injury that is due to reperfusion, with respect to that proportion that is due to ischemia itself (Figure 2). It appears that in many situations this proportion is large, and as a result, I believe that the therapeutic manipulation of free radical-mediated reperfusion will have a major role in the clinical armamentarium of the practicing physician in the near future.

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Discussion

Riley: On one of your slides you said 'flush with Euro-Collin's'.

Bulkley: 'Euro-Collin's' is a high potassium salt solution that is standard material that is used together with mannitol in transplantation surgery. The reason we did that particular experiment was that it has long been known that mannitol is a very good treatment for acute tubular necrosis and it is routine to include mannitol in the flushing solution and to give mannitol to the recipient at the time of reperfusion. The feeling has been that its benefit is because of its osmotic effects. As many people here know, mannitol has been widely used as an $OH^{\cdot}$ scavenger in free radical studies. This, therefore, raised the problem that we may already be getting a benefit anyway and when we went to apply free radical therapy in the clinic we might not see any effect.

Adams: Could I ask you about tumours? As you will have gathered a number of people here are interested in the role of oxygen deficiency in tumours. Traditionally it has been the radio resistance of the hypoxic cells which has been a possible limiting factor in local radiotherapy. Much more recently people have been interested in going in the other direction, in making tumours more hypoxic with the point of view of increasing the activation of bioreductive drugs. A technique that we use to do that is to employ a drug to shift the oxy-haemoglobin dissociation curve to the left. We find that it does make normal tissues somewhat hypoxic, for example by radiation monitoring. But some tumours show a substantial increase in necrosis.

Bulkley: Without the radiation?

Adams: With no radiation at all. To try to quantitate this, histopathologists looked at infiltrated lymph nodes and T-cell lymphoma in mice which are small and not normally necrotic. It was found that inducing this left shift, with a drug which had a quite short half life – about 18 minutes – caused large necrosis in these lymph nodes. In fact they shrank. If on the other hand a drug with a much longer life, say 24 hours, was used there was no effect. Allopurinol did not have any effect in this situation.